

# Alterations of Cytochrome P450-dependent Monooxygenase Activities in *Eriocheir japonicus* in Response to Water Pollution

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*Eriocheir japonicus*, fresh-water crabs inhabiting rivers and estuaries in Japan, were investigated for cytochrome P450 (CYP)-dependent drug-metabolizing enzyme activities to see if these activities reflect the river pollution gradient. From the laboratory dose-response experiments, we found that the polycyclic aromatic hydrocarbon (PAH) 3-methylcholanthrene induced total CYP contents, ethoxycoumarin *O*-deethylase activity, and bunitrolol 4-hydroxylase activity in crab hepatopancreas. In the field studies, crabs collected from the river with the highest concentration of PAHs exhibited the highest levels of CYP, the highest activities of benzo[*a*]pyrene 3-hydroxylase, imipramine 2-hydroxylase, bunitrolol 4-hydroxylase, ethoxycoumarin *O*-deethylase, and the ability to metabolically activate benzo[*a*]pyrene, but erythromycin *N*-demethylase activity was not induced. The correlation between PAH levels and drug-metabolizing enzyme activities in female crabs were not as marked as in male crabs. The levels and activities of CYP did not appear to reflect the concentrations of organochlorines and polychlorinated biphenyl congeners (PCBs) studied in the fat of crab hepatopancreas. **Key words:** crabs, cytochrome P450, environmental monitoring, *Eriocheir japonicus*, PCBs, polycyclic aromatic hydrocarbons. *Environ Health Perspect* 104:774-778 (1996)

The monooxygenase system, which metabolizes a number of foreign compounds, is widely distributed among organisms. The presence of cytochrome P450 (CYP), an important component of the drug-metabolizing enzyme system, has been reported in several aquatic species (1-5). Rivers and marine environments are contaminated with many lipophilic chemicals. Due to the low solubility of these compounds in water, ingestion of food on to which pollutants have been solubilized could be a major route of exposure to environmental contaminants in aquatic species. Fresh-water crabs are at the top of the food chain in the aquatic environment and are among the commonest animals in rivers and estuaries. In crabs, the hepatopancreas—a large, fatty gland—is the major organ of metabolism and digestion (6). The induction of some forms of CYP by many foreign chemicals has been reported in several crab species. Therefore, we investigated this induction phenomenon in crabs as a way to monitor water quality.

*Eriocheir japonicus* is a common crab species in Japan. We investigated alterations of CYP concentrations and drug-metabolizing activities in hepatopancreatic microsomes after injecting these crabs with a polycyclic aromatic hydrocarbon (PAH). 3-methylcholanthrene (3-MC) was selected as the inducing agent because in other species it is a potent and effective inducer of the forms of CYP that metabolize PAHs. Several studies have shown that the administration of 3-MC causes induction of CYP in the hepatopancreatic microsomes of crustaceans.

A field investigation was undertaken to test if the levels of pollution correlate with drug-metabolizing enzyme activities in crabs. The level of environmental pollution, including PAHs in the areas inhabited by crabs and the concentrations of organochlorines and polychlorinated biphenyl congeners (PCBs) in the crabs, was determined.

## Materials and Methods

**Animals and preparation of microsomes.** We collected adult male *Eriocheir japonicus* (230.0 ± 77.95 g) from the Barato River between August and September. The crabs were kept in glass aquariums and fed commercial pet food for 2 weeks before treatment with inducer. The crabs were given three oral intubations with different doses of 3-MC in corn oil 3 days before analysis (0.2, 1.0, 5.0, or 40.0 mg/kg/day). Control animals received three oral intubations of corn oil only (1.0 ml/kg/day). Crabs were sacrificed on the fourth day and microsomes were prepared.

For the field investigation, we collected crabs from the Ishikari Bay at the mouths of the Ishikari River, the Barato River, the Shiribetsu River, and the Tone River (Fig. 1). The Ishikari River has been contaminated with the waste of the paper mills. The Barato River is a branch of the Ishikari River and runs through a suburban agricultural area. The Shiribetsu River runs through a rural area and a mountain village. The Tone River flows through industrial, agricultural, residential, and urban areas and has been polluted with the wastes from

these areas. Crabs of both sexes were caught between July and August. Only adults in their intermoult stage were selected for this study. Animals were sacrificed immediately after collection. Male (94.14 ± 51.95 g) and female (85.49 ± 28.39 g) crabs were anesthetized by cooling in ice for 10-20 min and dissected as rapidly as possible.

Hepatopancreas was washed in buffer (0.05 M Tris, 1 mM EDTA, 2.5 mM dithiothreitol, 0.5 mM diisopropyl fluorophosphate, 20% glycerol, 50 µg/ml aprotinin, pH 7.4) and homogenized (7). Particular attention was paid to keeping the homogenate cold and keeping the protease inactive by freshly preparing protease inhibitor at each experiment. Microsomes were prepared from postmitochondrial supernatants by centrifugation at 105,000g. The microsomal pellet resulting from the centrifugation was resuspended in the same buffer and centrifuged again for 60 min at 105,000g. The washed microsomes were then suspended in the buffer. The pools of microsomal fraction, derived from three crabs, were stored at -80°C.

Fat fractions were prepared from supernatants by centrifugation at 800g and cleaned up using the method of Kannan et al. (8,9) with some modifications.

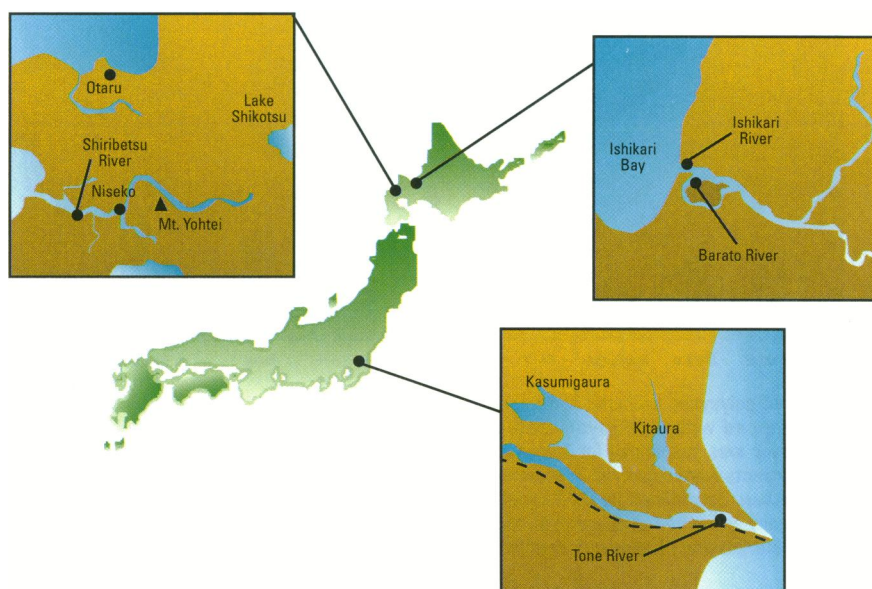
**Chemical analyses.** The PAHs were trapped with blue rayon using the method of Hayatsu et al. (10). Blue rayon, bearing copper phthalocyanine trisulfonate as a covalently linked ligand, is an adsorbent specific for compounds with three or more fused rings. Blue rayon (3 g) in plastic-mesh bags, 30 cm length × 5 cm diameter, were placed at the crab collection sites for three days during the time period crabs were collected.

We dissolved the extracts in acetonitrile, and determined PAHs by UV-fluorescence spectroscopy. The fluorescence of the acetonitrile extract was assayed with an excita-

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**Figure 1.** Map of Japan showing sampling locations.

tion wavelength of 384 nm and an emission wavelength of 406 nm, using benzo[*a*]pyrene as the standard.

Polychlorinated biphenyl (PCB) derivatives and insecticides in single-pool fat fraction from nine crabs were measured according to the method of Kannan et al. (8,9). PCB- and insecticide-containing fractions were analyzed using a gas chromatograph equipped with an  $^{63}\text{Ni}$  electron capture detector. The injector and the detector temperature were kept at 220°C and 320°C, respectively. The column temperature was programmed to rise from 160°C to 260°C at the rate of 2°C/min, maintaining the final temperature for 20 min.

**Biochemical assay.** We determined microsomal protein concentrations using the method of Lowry et al. (11). Levels of CYP were determined according to the method of Omura and Sato (12). Enzyme activities in microsomes were measured at an incubation temperature of 20°C in the presence of an NADPH-generating system. Reactions were carried out under optimal conditions of incubation temperature, protein concentration, and incubation time.

Ethoxycoumarin *O*-deethylase activity which is CYP1A1 and CYP1A2 dependent in rats, was assayed according to Greenlee and Poland (13). The 1-ml reaction mixture contained 2 mg microsomal protein. We assayed the fluorescence of 7-hydroxycoumarin with excitation wavelength at 368 nm and emission wavelength at 456 nm using a JASCO FP-777 spectrofluorometer (JASCO Ltd., Tokyo).

Imipramine 2-hydroxylase activity, which is CYP2D dependent in rats, was

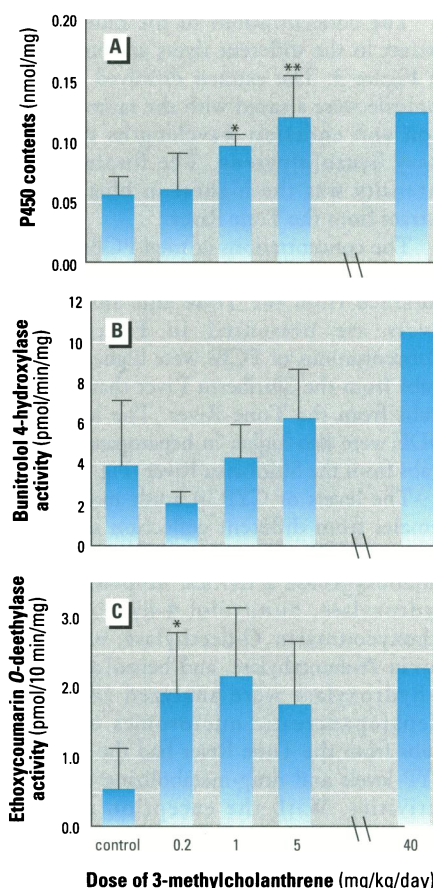
measured by HPLC as described previously (14). The assay mixture, containing a final concentration of 1 mg/ml microsomal protein and 1 mM substrate, was incubated for 5 min.

Bunitrolol 4-hydroxylase activity, which is also CYP2D dependent in rats, was determined by HPLC equipped with a fluorescence spectrophotometric detector (wavelength 325 nm and emission wavelength 365 nm) according to the method described by Ishida et al. (15). The incubation mixture for the assay contained a final concentration of 1 mg/ml microsomal protein and 1 mM substrate in a final volume of 1.0 ml.

Benzo[*a*]pyrene 3-hydroxylase activity, which is CYP1A1 and CYP1A2 dependent in rats, was measured using the method of Nebert and Gelboin (16). The fluorescence of the alkaline extract was assayed with an excitation wavelength of 396 nm and an emission wavelength of 522 nm. The concentrations of 3-hydroxybenzo[*a*]pyrene were calibrated with a quinine sulfate, and the values were converted using the factor given by Uemura and Chiesara (17).

Erythromycin *N*-demethylase activity, which is CYP3A dependent in rats, was measured by the method of Nash (19), with a final substrate concentration of 1 mM and a microsomal concentration of 160 µg/ml.

The mutagenesis assay was performed according to the method of Ames et al. (20) with modifications, using *Salmonella typhimurium* strains TA98 as the test strain and benzo[*a*]pyrene as the mutagen. The bacteria were preincubated with microsomes of crab hepatopancreas at 20°C.



**Figure 2.** Cytochrome P450 levels and metabolic activities in hepatopancreatic microsomes from male crabs treated with 3-methylcholanthrene (3-MC). Five groups of crabs, three per group (except two in 40 mg/kg/day treatment group), were given three oral intubations with different doses of 3-MC in corn oil 3 days before analysis (0.2, 1.0, 5.0, 40.0 mg/kg/day). Control animals received three oral intubations of corn oil only (1.0 ml/kg/day). \* $p < 0.05$ , \*\* $p < 0.01$ , control vs. treated crabs. (A) Cytochrome P450 levels, (B) bunitrolol 4-hydroxylase activity, (C) ethoxycoumarin *O*-deethylase activity.

## Results

Treatment of crabs with different doses of 3-MC resulted in induction of CYP related to drug metabolism (Fig. 2). CYP in hepatopancreatic microsomes showed a graded response to doses of 3-MC (Fig. 2A). Although statistically significant differences were not observed in comparing bunitrolol 4-hydroxylase activities among treated and control crabs (Fig. 2B), there were dose-dependent increases in treated animals. Ethoxycoumarin *O*-deethylase activity was significantly increased in treated animals (Fig. 2C). The level of activity was threefold high in crabs treated with 0.2 mg/kg/day, the lowest dose of 3-MC. The level of activity did not increase even at the 40 mg/kg/day, indicating that crab CYP was maximally induced at this lowest dose.



The concentrations of the blue rayon extract in the different rivers are presented in Figure 3. The extracts dissolved in acetonitrile were assayed with the same excitation and emission wavelengths used to assay benzo[a]pyrene. The fluorescence intensity was the highest in blue rayon extract from the Tone River.

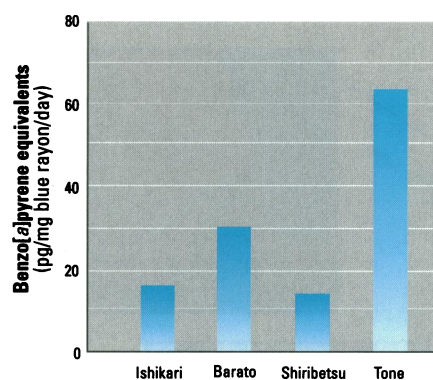
The concentrations of total PCBs in the fatty fraction of hepatopancreas of crabs obtained from the Tone and Shiribetsu rivers are presented in Figure 4A. Concentrations of PCBs were higher in the crabs from the Shiribetsu River than in the crabs from the Tone River. The levels of DDE were also higher in hepatopancreas of crabs from the Shiribetsu River (Fig. 4B).

The levels of CYP in adult males and females from different collection sites are shown in Figure 5A. CYP-mediated monooxygenase activities, imipramine 2-hydroxylase, bunitrolol 4-hydroxylase, ethoxycoumarin *O*-deethylase, erythromycin *N*-demethylase, and benzo[a]pyrene 3-hydroxylase were analyzed (Fig. 5). Hepatopancreatic microsomes of male crabs from the Tone River had the highest CYP levels and drug-metabolizing enzyme activities. With the exception of erythromycin *N*-demethylase activity, CYP levels and activities were not detected or were very low in male crabs from the Shiribetsu River. High activities of benzo[a]pyrene 3-hydroxylase were not observed for crabs from the Ishikari Bay, even though these crabs had relatively high CYP content. Except for bunitrolol 4-hydroxylation and imipramine 2-hydroxylation, we have not observed remarkably high drug-metabolizing activities in female crabs from the Tone River. Significant variations were not observed in the erythromycin *N*-demethylase activities in hepatopancreas of male or female crabs inhabiting different areas.

The abilities of metabolic activation of benzo[a]pyrene were investigated in hepatopancreatic microsomes from male crabs (Fig. 6). Crabs inhabiting the Tone River had the highest rates of activation. The lowest rates of mutagenic activation were observed in crabs from the Ishikari Bay.

## Discussion

CYP levels were significantly higher in hepatopancreas from 3-MC-treated crabs. Ethoxycoumarin *O*-deethylase activities were significantly increased in treated animals. Bunitrolol 4-hydroxylase activities tended to increase in treated animals. Possible explanations for the lack of greater increases in ethoxycoumarin *O*-deethylase activities in crabs treated with 5.0 or 40.0 mg 3-MC/kg/day are the toxicity of the

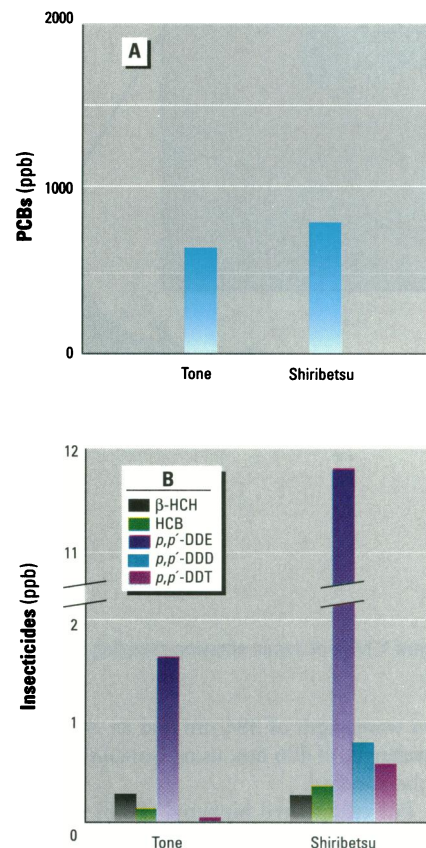


**Figure 3.** Levels of polycyclic aromatic hydrocarbons (PAHs) trapped with blue rayon. Three grams of blue rayon was suspended for 3 days in the rivers. The extracts were dissolved in acetonitrile, then PAHs were determined by UV-fluorescence spectroscopy, using benzo[a]pyrene as the standard. See Materials and Methods for details.

inducer or that these doses are greater than the amount needed to cause maximal induction. The form of CYP corresponding to bunitrolol 4-hydroxylase activity may be differentially affected.

We investigated the levels of PAHs in rivers to assess a possible correlation between contaminant levels in water and CYP enzyme activity of crabs inhabiting the same water. The work by Lee et al. (6) with blue crabs (*Callinectes sapidus*) demonstrated that crabs should not retain petroleum hydrocarbons due to the high rate of hydrocarbon metabolism and excretion. Therefore, we did not measure tissue concentrations of PAHs in the crabs but measured PAH concentrations in river water. The highest rates of metabolic activation, enzyme levels, and drug-metabolizing enzyme activities were observed in hepatopancreatic microsomes of crabs from the Tone River, which contained elevated levels of contaminants.

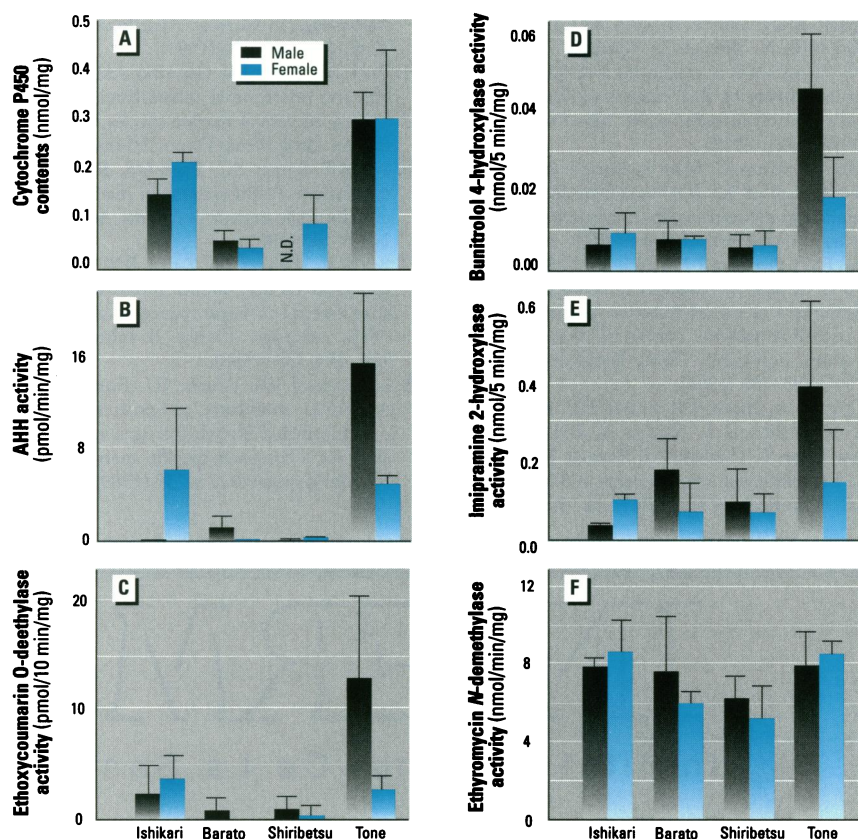
Recent studies have demonstrated the usefulness of ethoxyresorufin *O*-deethylase activity as an indicator of the level of environmental pollution (21,22). Our study showed that levels of male hepatopancreatic CYP enzymes, activities of benzo[a]pyrene 3-hydroxylase, ethoxycoumarin *O*-deethylase, imipramine 2-hydroxylase, bunitrolol 4-hydroxylase, and metabolic activation of benzo[a]pyrene were the highest in hepatopancreatic microsomes of crabs from the Tone River. Activities of these enzymes appear to also be useful indicators of levels of PAHs in environment. Erythromycin *N*-demethylase activity did not reflect the levels of contaminants. Hexobarbital hydroxylase activity and aniline 4-hydroxylase activity could not be detected in hepatopancreatic microsomes from *E. japonicus* (data not shown). Bunitrolol 4-hydroxylase and



**Figure 4.** Levels of polychlorinated biphenyls (PCBs) and insecticides from the fat fraction of crab hepatopancreas. PCB- and insecticides-containing fractions were injected into a gas chromatograph equipped with an electron capture detector. See Materials and Methods for details. (A) Concentration (ng/g fat) of PCB compounds in fat fraction, (B) concentration (ng/g fat) of DDT compounds, hexachlorocyclohexane (HCH), and hexachlorobenzene (HCB) in fat fraction.

imipramine 2-hydroxylase are mainly catalyzed by CYP2D1 in rat liver microsomes, and benzo[a]pyrene hydroxylase is catalyzed by CYP1A1 and CYP1A2 in microsomes from the induced liver and by CYP2C11 in microsomes from the noninduced liver. Ethoxycoumarin *O*-deethylase is catalyzed by CYP1A1 and CYP1A2 and erythromycin *N*-demethylase by CYP3A. In crabs, however, there is no information on multiple types of CYP. The classification of CYP types based on the enzymatic activities using different substrates in the hepatopancreas of crabs may not follow the rule applicable to vertebrates.

Porte et al. (23) reported correlations between CYP levels and tissue concentrations of PCBs in *Mytilus* sp. In highly polluted areas, ethoxyresorufin *O*-deethylase activity and CYP1A1 protein were induced in flatfish (24). Induction studies in several aquatic species suggest that increases can be produced by exposure to Aroclor 1254 and



**Figure 5.** Cytochrome P450 levels and metabolic activities in microsomes from hepatopancreas of male and female *E. japonicus*. The cytochrome P450 content was calculated from the carbon monoxide-difference spectra of reduced microsomes (72). Each value represents the means  $\pm$  SD from three animals. ND, not detectable. (A) Cytochrome P450 level, (B) benzo[a]pyrene 3-hydroxylase activity, (C) ethoxycoumarin O-deethylase activity, (D) bunitrolol 4-hydroxylase activity, (E) imipramine 2-hydroxylase activities, and (F) erythromycin N-demethylase activity.

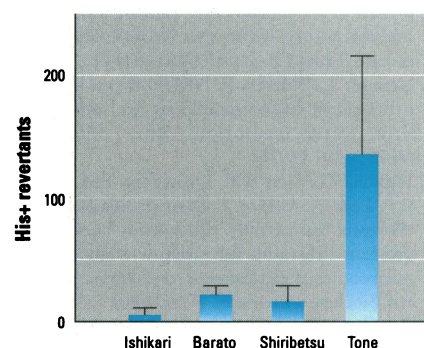
DDTs (25–27). However, in the present investigation, the levels of CYP and enzyme activities did not reflect the tissue levels of PCBs and insecticides. It is possible that highly induced crabs have lower levels of environmental contaminants in their tissues because the contaminants are rapidly metabolized by induced enzymes. Another possibility is that the PCB isomers and congeners, which were accumulated in the crabs, did not induce CYP and drug-metabolizing enzymes. PCBs consist of many isomers and congeners which have a variety of induction potencies toward CYP (28). Isomer-specific analysis of PCBs was not conducted in this investigation.

CYP levels in female crabs paralleled those in male crabs. In females, however, the fluorescence intensity of extracts from the water the crabs inhabited showed high correlation only with imipramine 2-hydroxylase activity. The enzyme activities of the hepatopancreatic microsomes of crabs from Tone River appear to indicate the presence of sex-specific differences in these activities. However, clear sex differences were observed only in crabs from

Tone River, the most polluted river studied. This may indicate that female crabs are less responsive to the induction of CYP; females may not be as sensitive as males to foreign chemicals in the induction of drug-metabolizing enzymes tested in this study. Female crabs may not accumulate pollutant chemicals to a high enough concentration to induce CYP because they lay large numbers of eggs, and the pollutants may be transferred to eggs. The male crabs collected from the Tone River, with high PAH concentrations, exhibited high CYP levels and drug-metabolizing activities.

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**Figure 6.** Metabolic activation of benzo[a]pyrene by microsomes from hepatopancreas of male *E. japonicus*. Mutagenicity was assayed using *S. typhimurium* TA98 (20).

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